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Reduction in Intensity of *Pneumocystis carinii* Pneumonia in Mice by Aerosol Administration of Gamma Interferon

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***Pneumocystis carinii* pneumonia in patients with AIDS may result from impaired local release of gamma interferon from lung lymphocytes and subsequent failure of macrophage activation. We tested this hypothesis with mice rendered immunodeficient by selective depletion of CD4$^+$ lymphocytes and inoculated intratracheally with *P. carinii*. After aerosol administration of recombinant murine gamma interferon, the intensity of *P. carinii* infection in these mice was reduced in comparison with that in mice not treated with gamma interferon. Histologic scoring of lung tissue did not reveal additional pulmonary inflammation attributable to gamma interferon. Aerosol administration of gamma interferon may reduce the intensity of *P. carinii* infection by augmentation of host defense, despite persistent CD4$^+$ lymphocyte depletion.**

Pneumocystis carinii pneumonia is an important cause of morbidity and mortality in patients with AIDS, but mechanisms of host defense against *P. carinii* are not well understood (11). Depletion of CD4$^+$ lymphocytes, a major immunologic abnormality in patients with AIDS, is correlated with an increased risk of development of *P. carinii* pneumonia (15). The CD4$^+$ lymphocyte is unlikely to function alone as an effector cell against *P. carinii*, but failure of CD4$^+$ lymphocytes to activate other immune effector cells, such as alveolar macrophages, may be responsible for establishment of this infection. Gamma interferon (IFN-γ), a product of CD4$^+$ lymphocytes, may be a deficient activating signal. Lymphocytes from patients with AIDS are deficient in their ability to produce IFN-γ after stimulation with antigen (14) or mitogen (17). However, alveolar macrophages (12) and blood monocytes (13) from patients with AIDS respond normally to IFN-γ in vitro.

We hypothesized that administration of IFN-γ to mice infected with *P. carinii* would decrease the intensity of infection. We examined this hypothesis in vivo by using a murine model of *P. carinii* infection in which mice are rendered immunodeficient by selective depletion of CD4$^+$ lymphocytes with injections of monoclonal antibody GK1.5 (20). After intratracheal inoculation with *P. carinii* organisms, these mice develop persistent pulmonary infection. We chose to administer IFN-γ by aerosol inhalation to provide site-specific cytokine delivery. In previous work, we have demonstrated that cytokines administered by aerosol inhalation, including IFN-γ, induce sustained enhancement of immunologic function of alveolar macrophages in rats (6). Furthermore, aerosol administration of IFN-γ to rats during corticosteroid immunosuppression increases Ia antigen expression on alveolar macrophages, which is a measure of cellular activation (8).

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MATERIALS AND METHODS

P. carinii infection was established in mice selectively depleted of CD4$^+$ lymphocytes as previously described (20). Virus-free, male BALB/c mice were obtained at 8 weeks of age (Simonsen Laboratories, Gilroy, Calif.) and were selectively depleted of CD4$^+$ lymphocytes by weekly injections of 0.3 mg of monoclonal antibody GK1.5 (rat IgG2b; American Type Culture Collection, Rockville, Md.). This antibody was purified from ascites produced by pristane-primed athymic mice. After two weekly injections of monoclonal antibody, mice received two weekly intratracheal inoculations with 2×10^5 *P. carinii* cysts in a volume of 100 μl during pentobarbital anesthesia. This dose was chosen because it results in reproducibly severe *P. carinii* infection 6 weeks after inoculation (2). The *P. carinii* inoculum was prepared from lung homogenates from athymic mice chronically infected with *P. carinii* (Fox Chase Cancer Center, Philadelphia, Pa.). After *P. carinii* inoculation, mice continued to receive weekly injections of monoclonal antibody.

Four weeks after *P. carinii* inoculation, test mice were exposed daily to aerosolized, recombinant murine IFN-γ (Genentech, South San Francisco, Calif.) for 2 weeks. Control mice received monoclonal antibody injections and *P. carinii* inoculations but were not exposed to the aerosol apparatus. Aerosolization of IFN-γ was performed as previously described (6), using IFN-γ (2 mg; specific activity, 5×10^6 U/mg) diluted in 6 ml of phosphate-buffered saline. Both IFN-γ and the diluent were sterile and pyrogen-free and contained less than 0.025 pg of lipopolysaccharide per ml by the *Limulus* amoebocyte assay. IFN-γ was aerosolized with an Acorn 1 nebulizer (Marquest Products, Englewood, Colo.) driven by compressed air at a flow rate of 15 liters/min. Mice were exposed to this aerosol in a nose-only aerosol chamber (Intox Products, Albuquerque, N.Mex.) until the entire volume of solution was nebulized over approximately 30 min. To determine the distribution of particle size generated with this apparatus, the aerosol was sampled as it entered the chamber with a seven-stage,

Mercer-style cascade impactor (Intox) as previously described (4). The apparatus generated particles with a mass median aerodynamic diameter of 1.8 ± 1.4 μm and produced a calculated concentration of IFN-γ in air of 4.8 μg/liter. The retained dose per mouse was estimated as the product of minute volume (0.02 liters of air per min), drug concentration (4.8 μg/liter of air), exposure time (30 min), and fractional deposition of the inhaled protein in the lung (12% of inhaled dose deposited) (16). Therefore, the estimated retained dose was 350 ng of IFN-γ per mouse per session, equivalent to a cumulative dose of 4.9 μg of IFN-γ per mouse.

Two days after the final aerosolization period, mice treated with aerosolized IFN-γ and control mice were exsanguinated during pentobarbital anesthesia. After cannulation of the trachea, lung lavage was performed with 0.5-ml aliquots of warmed, calcium- and magnesium-free phosphate-buffered saline, to a total volume of 11 ml. Lung lavages obtained from mice were centrifuged at 500 × *g* for 10 min at 4°C; then the cell pellets were washed twice in cold phosphate-buffered saline. Resuspended cells were counted in a hemacytometer. Lavaged cells were prepared for differential counting by gravity filtration (3). After 10^5 cells were washed onto nitrocellulose filters (Millipore, Bedford, Mass.), filters were fixed in 10% formalin solution and stained with Carazzi's hematoxylin-eosin (18). Blinded differential counts were performed on 300 cells per slide.

After lavage, lungs were inflated with 0.8 ml of air and then fixed with 0.8 ml of 10% formalin solution in phosphate-buffered saline. Fixed lungs were sectioned parasagittally and embedded in paraffin. Tissue blocks were sectioned at 5-μm thicknesses, and sections were stained with the Gomori methenamine silver stain and the hematoxylin-eosin stain for histologic evaluation. Lung sections were scored histologically as previously described and validated (2, 20), by a pathologist (H.D.L.) who lacked previous knowledge of treatment or group assignment. A minimum of five segments of lung from each mouse were examined. The intensity of *P. carinii* infection was scored on a semiquantitative scale ranging from 1 (rare, individual organisms) to 4 (numerous organisms in every 40× field). The severity of inflammatory cell accumulation in perivascular, peribronchial, and alveolar areas was scored separately, on a semiquantitative scale ranging from 1 (very slight and sparsely scattered inflammatory cell accumulation) to 4 (large numbers of inflammatory cells in a generalized distribution). All data are presented as the mean ± standard error of the mean. Scalar comparisons were made by the *t* test, and ordinal comparisons were made by the Mann-Whitney test (23). Significance was accepted for $P < 0.05$.

RESULTS

We found that the intensity of *P. carinii* infection in mice treated with aerosolized IFN-γ was significantly reduced compared with that in control mice (Table 1; $P < 0.05$ by Mann-Whitney test). Of 10 mice treated with aerosolized IFN-γ, 4 showed no evidence of residual *P. carinii* cysts, whereas all 7 control mice showed cyst scores indicating moderate to severe infection (grade 2 or greater). Histologic scoring for perivascular, peribronchial, and alveolar inflammations did not differ between mice treated with aerosolized IFN-γ and control mice (Table 1 and Fig. 1). Total numbers of cells in lung lavages of mice treated with aerosolized IFN-γ ([8.7 ± 1.6] × 10^6 for 10 mice) were decreased compared with those of control mice ([10.2 ± 2.1] × 10^6 for 7 mice), although this difference did not attain statistical significance. The difference in total cell counts was a result of decreased numbers of lymphocytes in mice treated with aerosolized IFN-γ compared with control mice ([4.8 ± 1.0] × 10^6 versus [6.8 ± 1.6] × 10^6, respectively), rather than changes in numbers of macrophages ([1.9 ± 0.3] × 10^6 versus [1.6 ± 0.3] × 10^6) or neutrophils ([2.0 ± 0.6] × 10^6 versus [1.8 ± 0.7] × 10^6).

TABLE 1. Histologic scoring of lung sections from mice inoculated with *P. carinii*

Characteristic	Score for group[a]	
	Control	Aerosolized IFN-γ
Intensity of *P. carinii* infection	3.29 ± 0.29	1.80 ± 0.46[b]
Perivascular inflammation	2.00 ± 0.00	2.20 ± 0.44
Peribronchial inflammation	1.57 ± 0.37	1.20 ± 0.25
Alveolar inflammation	2.43 ± 0.30	2.00 ± 0.39

[a] Data are means ± standard errors for 7 mice in the control group and 10 mice in the group given aerosolized IFN-γ. Three mice from the control group died soon after inoculation with *P. carinii* and were excluded from further analysis. Lung sections were scored as described in the text.
[b] $P < 0.05$ versus control by the Mann-Whitney test (23).

DISCUSSION

This study shows that aerosolized IFN-γ reduces the intensity of *P. carinii* infection in mice, despite continued $CD4^+$ lymphocyte depletion. Our results confirm and extend a recent report of enhanced treatment of *P. carinii* infection in steroid-immunosuppressed rats given systemic IFN-γ, either by intraperitoneal infection or by implantation of an osmotic pump (19). However, the present study differs in three important aspects. First, the current study shows a positive effect of IFN-γ during a form of immunosuppression that more closely resembles the major immunologic defect in AIDS than does nonspecific corticosteroid administration. Second, we administered IFN-γ by a site-specific delivery method rather than by systemic administration to potentially avoid or reduce systemic toxicity (6). Third, we investigated inflammatory effects of IFN-γ in the lung and found no additional pulmonary inflammation attributable to IFN-γ. Histologic scoring of inflammation was comparable in mice treated with aerosolized IFN-γ and in control mice, and cell counts in lavages were lower for the mice treated with aerosolized IFN-γ than for the control mice. The absence of an inflammatory effect of aerosolized IFN-γ per se agrees with previous data showing minimal histologic inflammation in rats after exposure to aerosolized IFN-γ (6).

The treatment effect of IFN-γ in these experiments was likely due to a direct effect of cytokine rather than to repetitive inhalation of the saline diluent. Previous work from our laboratory has shown that administration of sham aerosol (inert buffer) to immunosuppressed rats with *P. carinii* infection does not have intrinsic activity against *P. carinii*, since the intensity of *P. carinii* infection in sham-exposed rats is identical to that of untreated rats (5). It is also unlikely that exposure to aerosolized saline resulted in pulmonary inflammation or in modulation of cellular activity. Neither the lung histology nor the numbers of cells obtained by bronchoalveolar lavage are altered by sham-aerosol exposure (6). Exposure of normal rats to aerosolized saline buffer does not increase alveolar macrophage production of interleukin-1 or expression of Ia antigen, whereas exposure to aerosolized IFN-γ produces both these effects

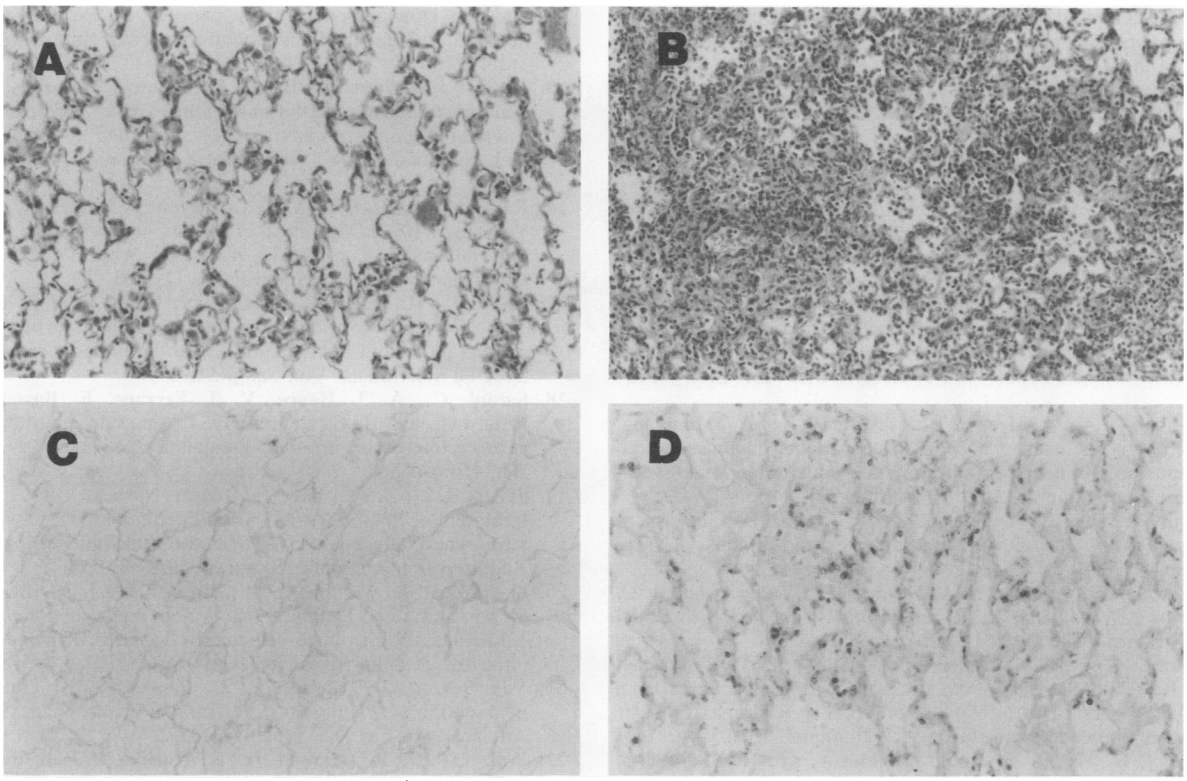

FIG. 1. Photomicrographs of lung sections from mice inoculated with *P. carinii*. (A) Grade 1 inflammatory changes. Alveoli contain a few macrophages, but significant numbers of perivascular or peribronchial inflammatory cells are not present (hematoxylin-eosin stain; magnification, ×50). (B) Grade 4 inflammatory changes. Large numbers of macrophages are present in alveoli, and accumulation of perivascular and peribronchial inflammatory cells is dense (hematoxylin-eosin stain; magnification, ×25). (C) Grade 1 *P. carinii* intensity (section from same mouse as that used for panel A). Sparsely distributed cysts are present (Gomori methenamine silver stain; magnification, ×50). (D) Grade 4 *P. carinii* intensity (section from same mouse as that used for panel B). Numerous cysts are present in aggregates (Gomori methenamine silver stain; magnification, ×50).

(6). Similarly, exposure of rats immunosuppressed with corticosteroids to saline aerosol does not result in immunomodulation of alveolar macrophages, but a single aerosol exposure to IFN-γ increases Ia antigen expression (8). Finally, both the IFN-γ and the diluent we administered were virtually free of endotoxin. Taken in aggregate, it is likely that IFN-γ rather than the saline decreased the intensity of *P. carinii* infection.

The likely target for aerosolized IFN-γ is the alveolar macrophage, although the role of this cell in host defense against *P. carinii* remains uncertain. Aerosolized IFN-γ reaches the alveolar space in concentrations sufficient to modulate the function of alveolar macrophages, as it increases Ia antigen on alveolar macrophages from rats similarly treated (8). In vivo, the numbers of alveolar macrophages increase in rats during withdrawal from corticosteroids and clearance of *P. carinii* infection (22). In our previous work, we have shown that inoculation of immunologically intact mice with *P. carinii* results in an increase in alveolar macrophages, which then declines as organisms are cleared (2). In vitro, phagocytosis of *P. carinii* organisms by alveolar and peritoneal macrophages has been demonstrated (10, 21). IFN-γ may augment macrophage defense against *P. carinii* by augmenting the production of superoxide metabolites (7), by increasing synthesis of inorganic nitrogen oxides (1), or by priming macrophages for tumor necrosis factor release (9).

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